# METHODS FOR PRODUCING POLYPEPTIDES IN CYCLOHEXADEPSIPEPTIDE-DEFICIENT CELLS

## Cross-Reference to Related Application

This application is a continuation-in-part of pending U.S. application Serial No. 09/229,862 filed January 13, 1999, which application is fully incorporated herein by reference.

## Background of the Invention

### Field of the Invention

The present invention relates to methods for producing heterologous polypeptides in cyclohexadepsipeptide-deficient filamentous fungal mutant cells. The present invention also relates to mutants of filamentous fungal cells and methods for obtaining the mutant cells. The present invention also relates to isolated cyclohexadepsipeptide synthetases and isolated nucleic acid sequences encoding the cyclohexadepsipeptide synthetases. The present invention also relates to nucleic acid constructs, vectors, and host cells comprising the nucleic acid sequences as well as methods for producing the cyclohexadepsipeptide synthetases. The present invention further relates to cyclohexadepsipeptides produced by the cyclohexadepsipeptide synthetases.

### Description of the Related Art

Depsipeptides constitute a large class of peptide-related compounds derived from hydroxy and amino acids joined by amide and ester linkages. Many members of this class of compounds are biologically active and include antibiotics, alkaloids, and proteins (Shemyakin *et al.*, 1969, *Journal of Membrane Biology* 1: 402-430). Examples include the enniatins, beauvericin, and bassianolide.

Enniatins are cyclohexadepsipeptide phytoxins with ionophoretic properties produced by various species of actinomycetes and filamentous fungi, particularly strains of *Fusarium.* They are composed of alternating D-2-hydroxyisovaleric acid residues and L-amino acids or

N-methyl-L-amino acids to form an 18-membered cyclic structure and may contain more than one species of amino acid.

The biosynthesis of enniatins is catalyzed by enniatin synthetase, which is a large multifunctional enzyme that has all the essential functions for assembling enniatins from their primary precursors, *i.e.*, D-2-hydroxyisovaleric acid, a branched chain L-amino acid (*e.g.*, valine, leucine, isoleucine), S-adenosylmethionine, and ATP (Reper *et al.*, 1995, *European Journal of Biochemistry* 230: 119-126). The precursors (D-2-hydroxyisovaleric acid and branched chain L-amino acid) are activated as thioesters. Covalently bound substrate amino acid residues are methylated under the consumption of S-adenosylmethionine. Then peptide bond formation and cyclization reactions occur.

Enniatins are postulated to play a role in wilt toxic events during infection by enniatin-producing fusaria (Walton, 1990, *Biochemistry of Peptide Antibiotics*, H. Kleinkauf and H. von Dohren, editors, W. de Gruytre, Berlin, pp. 179-203), and also exhibit entomopathogenic properties (Grove and Pople, 1980, *Mycopathologia* 70: 103-105).

The enniatin synthetase gene (*esyn1*) has been cloned from *Fusarium scirpi* (Haese *et al.*, 1993, *Molecular Microbiology* 7: 905-914).

Enniatin synthetase mutants of *Fusarium avenaceum* have been generated that do not produce enniatins (Herrmann *et al.*, 1996, *Molecular Plant-Microbe Interactions* 9: 226-232).

It is an object of the present invention to provide methods for producing heterologous polypeptides in cyclohexadepsipeptide-deficient filamentous fungal mutant cells.

## Summary of the Invention

The present invention relates to methods for producing a heterologous polypeptide, comprising: (a) cultivating a mutant of a parent filamentous fungal cell under conditions conducive for the production of the heterologous polypeptide, wherein (i) the mutant cell comprises a first nucleic acid sequence encoding the heterologous polypeptide, and (ii) the mutant produces less of a cyclohexadepsipeptide than the parent filamentous fungal cell when cultured under the same conditions; and (b) isolating the heterologous polypeptide from the cultivation medium.

The present invention also relates to mutants of filamentous fungal cells and methods

for obtaining the mutant cells.

The present invention also relates to isolated cyclohexadepsipeptide synthetases from *Fusarium venenatum* and isolated nucleic acid sequences encoding the cyclohexadepsipeptide synthetases. The invention also relates to nucleic acid constructs, vectors, and host cells comprising the nucleic acid sequences as well as methods for producing the cyclohexadepsipeptide synthetases.

The present invention further relates to cyclohexadepsipeptides produced by the cyclohexadepsipeptide synthetases.

## Brief Description of Figure

Figure 1 shows the genomic nucleic acid sequence and the deduced amino acid sequence of a *Fusarium venenatum* ATCC 20334 cyclohexadepsipeptide synthetase (SEQ ID NOS: 1 and 2, respectively).

Figure 2 shows the construction of pΔES-*amdS*.

## Detailed Description of the Invention

The present invention relates to methods for producing a heterologous polypeptide, comprising: (a) cultivating a mutant of a parent filamentous fungal cell under conditions conducive for the production of the heterologous polypeptide, wherein (i) the mutant filamentous fungal cell comprises a first nucleic acid sequence encoding the heterologous polypeptide and (ii) the mutant produces less of a cyclohexadepsipeptide than the parent filamentous fungal cell when cultured under the same conditions; and (b) isolating the heterologous polypeptide from the cultivation medium of the mutant cell.

The term "cyclohexadepsipeptide" is defined herein as a family of peptide-related compounds composed of hydroxy and amino acids linked by amide and ester bonds.

The term "production of a cyclohexadepsipeptide" is defined herein as to include any step involved in the production of a cyclohexadepsipeptide including, but not limited to, biosynthesis, regulation of biosynthesis, transport, and secretion.

In a preferred embodiment, the cyclohexadepsipeptide is an enniatin.

The term "enniatins" is defined herein as a family of cyclohexadepsipeptides

composed of three D-2-hydroxyisovaleric acid residues joined alternatively to L-amino acids or N-methyl-L-amino acids to produce an 18-membered cyclic structure. The enniatins include, but are not limited to, enniatin A, $A_1$, B, $B_1$, $B_2$, $B_3$, $B_4$, C, D, E, and F; and derivatives thereof (Visconte *et al.*, 1992, *Journal of Agricultural and Food Chemistry* 40: 1076-1082; Tomodo *et al.*, 1992, *Journal of Antibiotics* 45: 1207-1215), and mixed-type enniatins containing more than one species of amino acid (Zocher *et al.* 1982, *Biochemistry* 21: 43-48).

In the methods of the present invention, the filamentous fungal cell may be a wild-type cell or a mutant thereof. Furthermore, the filamentous fungal cell may be a cell that does not produce any detectable cyclohexadepsipeptide(s), but contains the genes encoding the cyclohexadepsipeptide(s). Preferably, the filamentous fungal cell is an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Beauveria*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Gibberella*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Myrothecium*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Piromyces*, *Polyporus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, or *Trichoderma* cell.

In a preferred embodiment, the filamentous fungal cell is an *Aspergillus aculeatus*, *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, or *Aspergillus oryzae* cell.

In another preferred embodiment, the filamentous fungal cell is a *Fusarium acuminatum*, *Fusarium avenaceum*, *Fusarium bactridioides*, *Fusarium compactum*, *Fusarium crookwellense* (synonym of *Fusarium cerealis*), *Fusarium culmorum*, *Fusarium equiseti*, *Fusarium gibbosum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium lateritium*, *Fusarium moniliforme*, *Fusarium negundi*, *Fusarium nivale*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium scirpi*, *Fusarium semitectum*, *Fusarium solani*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium tricinctum*, or *Fusarium venenatum* cell.

In another preferred embodiment, the filamentous fungal cell is a *Gibberella pulicaris*, *Gibberella zeae*, *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Myrothecium roridin*, *Neurospora crassa*, *Paeciliomyces fumoso-roseus*, *Penicillium purpurogenum*, or *Polyporus sulphureus* cell.

In another preferred embodiment, the filamentous fungal cell is a *Trichoderma*

*harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cell.

In a more preferred embodiment, the *Fusarium venenatum* cell is *Fusarium venenatum* A3/5, which was originally deposited as *Fusarium graminearum* ATCC 20334 and recently reclassified as *Fusarium venenatum* by Yoder and Christianson, 1998, *Fungal Genetics and Biology* 23: 62-80 and O'Donnell *et al.*, 1998, *Fungal Genetics and Biology* 23: 57-67; as well as taxonomic equivalents of *Fusarium venenatum* regardless of the species name by which they are currently known. In another more preferred embodiment, the *Fusarium venenatum* cell is a morphological mutant of *Fusarium venenatum* A3/5 or *Fusarium venenatum* ATCC 20334, as disclosed in WO 97/26330.

The filamentous fungal cell may also be a cell involved in the production of products containing (parts of) the mycelium, for example, in the production of the product QUORN™ (Marlow Foods, Ltd., Great Britain), which is produced from a *Fusarium* strain.

In the methods of the present invention, the mutant cell comprises a second nucleic acid sequence which comprises a modification of at least one of the genes involved in the production of the cyclohexadepsipeptide. Any gene of a filamentous fungal cell involved in the production of a cyclohexadepsipeptide may be modified. In a preferred embodiment, the gene is a cylcohexadepsipeptide synthetase gene. In a more preferred embodiment, the gene is an enniatin synthetase gene. In another more preferred embodiment, the gene is a D-hydroxyisovalerate dehydrogenase gene. D-Hydroxyisovalerate dehydrogenase catalyzes the conversion of 2-ketoisovalerate to D-hydroxyisovalerate (Lee and Zocher, 1996, *Journal of Biochemistry and Molecular Biology* 29: 493-499). In an even more preferred embodiment, the gene is a *Fusarium venenatum* cyclohexadepsipeptide synthetase gene having (a) a nucleic acid sequence encoding a polypeptide having an amino acid sequence which has at least 65% identity with the mature polypeptide contained within SEQ ID NO:2; (b) a nucleic acid sequence having at least 65% homology with the mature polypeptide coding region of SEQ ID NO:1; (c) a nucleic acid sequence which hybridizes under medium stringency conditions with (i) the nucleic acid sequence of SEQ ID NO:1, (ii) the cDNA sequence of SEQ ID NO:1, (iii) a subsequence of (i) or (ii) of at least 100 nucleotides, or (iv) a complementary strand of (i), (ii), or (iii); (d) an allelic variant of (a), (b), or (c); or (e) a subsequence of (a), (b), (c), or (d), wherein the subsequence encodes a polypeptide fragment which has cyclohexadepsipeptide synthetase activity. In a most preferred embodiment, the

gene is a *Fusarium venenatum* cyclohexadepsipeptide synthetase gene having the nucleic acid sequence of SEQ ID NO:1.

The cyclohexadepsipeptide-deficient filamentous fungal mutant cell may be constructed by reducing or eliminating expression of one or more of the genes described above using methods well known in the art, for example, insertions, disruptions, replacements, or deletions. The gene to be modified or inactivated may be, for example, the coding region or a part thereof essential for activity, or a regulatory element of the gene required for the expression of the coding region. An example of such a regulatory or control sequence may be a promoter sequence or a functional part thereof, *i.e.*, a part that is sufficient for affecting expression of the nucleic acid sequence. Other control sequences for possible modification include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, signal peptide sequence, transcription terminator, and transcriptional activator.

Modification or inactivation of the gene may be performed by subjecting the parent cell to mutagenesis and selecting for mutant cells in which expression of the gene has been reduced or eliminated. The mutagenesis, which may be specific or random, may be performed, for example, by use of a suitable physical or chemical mutagenizing agent, by use of a suitable oligonucleotide, or by subjecting the DNA sequence to PCR generated mutagenesis. Furthermore, the mutagenesis may be performed by use of any combination of these mutagenizing agents.

Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues.

When such agents are used, the mutagenesis is typically performed by incubating the parent cell to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions, and screening and/or selecting for mutant cells exhibiting reduced or no expression of the gene.

Modification or inactivation of the gene may be accomplished by introduction, substitution, or removal of one or more nucleotides in the gene or a regulatory element required for the transcription or translation thereof. For example, nucleotides may be inserted or removed so as to result in the introduction of a stop codon, the removal of the start codon, or a change in the open reading frame. Such modification or inactivation may be

accomplished by site-directed mutagenesis or PCR generated mutagenesis in accordance with methods known in the art. Although, in principle, the modification may be performed *in vivo*, *i.e.*, directly on the cell expressing the gene to be modified, it is preferred that the modification be performed *in vitro* as exemplified below.

An example of a convenient way to eliminate or reduce production of a cyclohexadepsipeptide by a filamentous fungal cell of choice is based on techniques of gene replacement, gene deletion, or gene disruption. For example, in the gene disruption method, a nucleic acid sequence corresponding to the endogenous gene or gene fragment of interest is mutagenized *in vitro* to produce a defective nucleic acid sequence which is then transformed into the parent cell to produce a defective gene. By homologous recombination, the defective nucleic acid sequence replaces the endogenous gene or gene fragment. It may be desirable that the defective gene or gene fragment also encodes a marker that may be used for selection of transformants in which the nucleic acid sequence has been modified or destroyed. In a particularly preferred embodiment, the gene is disrupted with a selectable marker such as those described herein.

Alternatively, modification or inactivation of the gene may be performed by established anti-sense techniques using a nucleotide sequence complementary to the nucleic acid sequence of the gene. More specifically, expression of the gene by a filamentous fungal cell may be reduced or eliminated by introducing a nucleotide sequence complementary to the nucleic acid sequence of the gene that may be transcribed in the cell and is capable of hybridizing to the mRNA produced in the cell. Under conditions allowing the complementary anti-sense nucleotide sequence to hybridize to the mRNA, the amount of protein translated is thus reduced or eliminated.

A nucleic acid sequence complementary or homologous to the nucleic acid sequence of a gene involved in the production of a cyclohexadepsipeptide may be obtained from other microbial sources that produce cyclohexadepsipeptides.

Preferred sources for an enniatin synthetase gene having a nucleic acid sequence complementary or homologous to the nucleic acid sequence of SEQ ID NO:1 of *Fusarium venenatum* include other *Fusarium* strains. A more preferred source is *Fusarium scirpi* (Haese *et al.*, 1993, *supra*).

Preferred sources for D-hydroxyisovalerate dehydrogenase genes that may be complementary or homologous to the nucleic acid sequence of the corresponding genes of a

filamentous fungal cell include other *Fusarium* strains. A more preferred source for the D-hydroxyisovalerate dehydrogenase gene is *Fusarium sambucinum* (Lee and Zocher, 1996, *supra*). Furthermore, the nucleic acid sequences may be native to the filamentous fungal cell.

The level of cyclohexadepsipeptides produced by a mutant filamentous fungal cell of the present invention may be determined using the method of Visconti *et al.*, 1992, *Journal of Agriculture and Food Chemistry* 40: 1076-1082. Specifically, one ml of *Fusarium venenatum* cell-free culture broth is extracted twice with 2.0 ml ethyl acetate. The combined organic extracts are evaporated to dryness under a stream of nitrogen gas and redissolved in 0.5 ml hexane. One microliter samples are analyzed using a Hewlett-Packard 6890 GC/Series MSD system operating in the electron impact (EI) mode. Samples are injected on-column and separated utilizing a DB-5 capillary column (30 m x 0.25 mm, 0.25 μm film) employing a temperature program with heating from 120 to 300°C at a rate of 15°C/min. For example, enniatins A, A1, B, B1, B2 and B3 are identified by *m/z* ratios for the ($M^+$ + H) ion of 682, 668, 640, 654, 626 and 612, respectively.

The mutant filamentous fungal cell preferably produces at least about 25% less, more preferably at least about 50% less, even more preferably at least about 75% less, most preferably at least about 95% less, and even most preferably no cyclohexadepsipeptide than the corresponding parent filamentous fungal cell when cultured under identical conditions. The parent and mutant cells may be compared with regard to production of a cyclohexadepsipeptide under conditions conducive for the production of a polypeptide of interest or under conditions conducive for the production of a cyclohexadepsipeptide.

In another aspect of the present invention, the mutant filamentous fungal cell may additionally contain modifications of one or more third nucleic acid sequences that encode proteins that may be detrimental to the production, recovery, and/or application of the heterologous polypeptide of interest. The modification reduces or eliminates expression of the one or more third nucleic acid sequences resulting in a mutant cell that may produce more of the heterologous polypeptide than the mutant cell without the modification of the third nucleic acid sequence when cultured under the same conditions. The third nucleic acid sequence may encode any protein or enzyme. For example, the enzyme may be an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, invertase, laccase,

lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phospholipase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, or xylanase. The third nucleic acid sequence preferably encodes a proteolytic enzyme, *e.g.*, an aminopeptidase, carboxypeptidase, or endoprotease.

The mutant filamentous fungal cell is cultivated in a nutrient medium suitable for production of a heterologous polypeptide of interest using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the heterologous polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (*e.g.*, in catalogues of the American Type Culture Collection). The secreted heterologous polypeptide can be recovered directly from the medium.

The heterologous polypeptide may be detected using methods known in the art that are specific for the polypeptide. These detection methods may include use of specific antibodies, formation of an enzyme product, disappearance of an enzyme substrate, or SDS-PAGE. For example, an enzyme assay may be used to determine the activity of the heterologous polypeptide. Procedures for determining enzyme activity are known in the art for many enzymes.

The resulting heterologous polypeptide may be isolated by methods known in the art. For example, the polypeptide may be isolated from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation. The isolated polypeptide may then be further purified by a variety of procedures known in the art including, but not limited to, chromatography (*e.g.*, ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (*e.g.*, preparative isoelectric focusing), differential solubility (*e.g.*, ammonium sulfate precipitation), or extraction (see, *e.g.*, *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

The polypeptide may be any polypeptide heterologous to the mutant filamentous fungal cell. The term "polypeptide" is not meant herein to refer to a specific length of the

encoded product and, therefore, encompasses peptides, oligopeptides, and proteins. The term "heterologous polypeptide" is defined herein as a polypeptide that is not native to the fungal cell, a native protein in which modifications have been made to alter the native sequence, or a native protein whose expression is quantitatively altered as a result of a manipulation of the fungal cell by recombinant DNA techniques. The mutant fungal cell may contain one or more copies of the nucleic acid sequence encoding the polypeptide. In a preferred embodiment, the heterologous polypeptide is an extracellularly secreted polypeptide.

Preferably, the heterologous polypeptide is a hormone, hormone variant, enzyme, receptor or portion thereof, antibody or portion thereof, or reporter. In a more preferred embodiment, the heterologous polypeptide is an oxidoreductase, transferase, hydrolase, lyase, isomerase, or ligase. In an even more preferred embodiment, the heterologous polypeptide is an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phospholipase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase or xylanase.

The nucleic acid sequence encoding a heterologous polypeptide that can be expressed in a filamentous fungal cell may be obtained from any prokaryotic, eukaryotic, or other source. For purposes of the present invention, the term "obtained from" as used herein in connection with a given source shall mean that the polypeptide is produced by the source or by a cell in which a gene from the source has been inserted.

In the methods of the present invention, the mutant filamentous fungal cell may also be used for the recombinant production of polypeptides that are native to the cell. The native polypeptides may be recombinantly produced by, *e.g.*, placing a gene encoding the polypeptide under the control of a different promoter to enhance expression of the polypeptide, to expedite export of a native polypeptide of interest outside the cell by use of a signal sequence, and to increase the copy number of a gene encoding the polypeptide normally produced by the cell. The present invention also encompasses, within the scope of the term "heterologous polypeptide", such recombinant production of homologous polypeptides, to the extent that such expression involves the use of genetic elements not native to the cell, or use of native elements that have been manipulated to function in a manner that do not normally occur in the host cell.

The techniques used to isolate or clone a nucleic acid sequence encoding a heterologous polypeptide are known in the art and include isolation from genomic DNA, preparation from cDNA, or a combination thereof. The cloning of the nucleic acid sequence from such genomic DNA can be effected, *e.g.*, by using the well known polymerase chain reaction (PCR). See, for example, Innis *et al.*, 1990, *PCR Protocols: A Guide to Methods and Application*, Academic Press, New York. The cloning procedures may involve excision and isolation of a desired nucleic acid fragment comprising the nucleic acid sequence encoding the polypeptide, insertion of the fragment into a vector molecule, and incorporation of the recombinant vector into the mutant fungal cell where multiple copies or clones of the nucleic acid sequence will be replicated. The nucleic acid sequence may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

In the methods of the present invention, heterologous polypeptides may also include fused or hybrid polypeptides in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide or fragment thereof. A fused polypeptide is produced by fusing a nucleic acid sequence (or a portion thereof) encoding one polypeptide to a nucleic acid sequence (or a portion thereof) encoding another polypeptide. Techniques for producing fusion polypeptides are known in the art, and include, ligating the coding sequences encoding the polypeptides so that they are in frame and expression of the fused polypeptide is under control of the same promoter(s) and terminator. The hybrid polypeptides may comprise a combination of partial or complete polypeptide sequences obtained from at least two different polypeptides wherein one or more may be heterologous to the mutant filamentous fungal cell.

An isolated nucleic acid sequence encoding a heterologous polypeptide of interest may be manipulated in a variety of ways to provide for expression of the polypeptide. Expression will be understood to include any step involved in the production of the polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion. Manipulation of the nucleic acid sequence prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying nucleic acid sequences utilizing cloning methods are well known in the art.

"Nucleic acid construct" is defined herein as a nucleic acid molecule, either single- or double-stranded, isolated from a naturally occurring gene or modified to contain segments of nucleic acid that are combined and juxtaposed in a manner which would not otherwise exist

in nature. The term nucleic acid construct is synonymous with the term expression cassette when the nucleic acid construct contains all the control sequences required for expression of a coding sequence. The term "coding sequence" as defined herein is a sequence that is transcribed into mRNA and translated into a polypeptide. The boundaries of the coding sequence are generally determined by the ATG start codon located just upstream of the open reading frame at the 5' end of the mRNA and a transcription terminator sequence located just downstream of the open reading frame at the 3' end of the mRNA. A coding sequence can include, but is not limited to, genomic, cDNA, RNA, semisynthetic, synthetic, recombinant, or any combinations thereof.

The term "control sequences" is defined herein to include all components that are necessary or advantageous for the expression of a heterologous polypeptide. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide. Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the nucleic acid sequence encoding a heterologous polypeptide. The term "operably linked" is defined herein as a configuration in which a control sequence is appropriately placed at a position relative to the coding sequence of the DNA sequence such that the control sequence directs the production of a heterologous polypeptide.

The control sequence may be an appropriate promoter sequence, a nucleic acid sequence that is recognized by a filamentous fungal cell for expression of the nucleic acid sequence. The promoter sequence contains transcriptional control sequences that mediate the expression of the heterologous polypeptide. The promoter may be any nucleic acid sequence that shows transcriptional activity in the filamentous fungal cell including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the cell.

Examples of suitable promoters for directing the transcription of the nucleic acid constructs in the methods of the present invention are promoters obtained from the genes encoding *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase,

*Aspergillus niger* or *Aspergillus awamori* glucoamylase (*glaA*), *Rhizomucor miehei* lipase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Aspergillus nidulans* acetamidase, *Aspergillus oryzae* acetamidase (*amdS*), *Fusarium oxysporum* trypsin-like protease (U.S. Patent No. 4,288,627), and mutant, truncated, and hybrid promoters thereof. Particularly preferred promoters are the NA2-tpi (a hybrid of the promoters from the genes encoding *Aspergillus niger* neutral alpha-amylase and *Aspergillus oryzae* triose phosphate isomerase), glucoamylase, and TAKA amylase promoters.

The control sequence may also be a suitable transcription terminator sequence, a sequence recognized by a filamentous fungal cell to terminate transcription. The terminator sequence is operably linked to the 3' terminus of the nucleic acid sequence encoding the heterologous polypeptide. Any terminator that is functional in the filamentous fungal cell may be used in the present invention.

Preferred terminators are obtained from the genes encoding *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthetase, *Aspergillus niger* alpha-glucosidase, and *Fusarium oxysporum* trypsin-like protease.

The control sequence may also be a suitable leader sequence, a nontranslated region of a mRNA that is important for translation by the filamentous fungal cell. The leader sequence is operably linked to the 5' terminus of the nucleic acid sequence encoding the heterologous polypeptide. Any leader sequence that is functional in the filamentous fungal cell may be used in the present invention.

Preferred leaders are obtained from the genes encoding *Aspergillus oryzae* TAKA amylase and *Aspergillus nidulans* triose phosphate isomerase.

The control sequence may also be a polyadenylation sequence, a sequence operably linked to the 3' terminus of the nucleic acid sequence and, when transcribed, is recognized by a filamentous fungal cell as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence that is functional in the filamentous fungal cell may be used in the present invention.

Preferred polyadenylation sequences are obtained from the genes encoding *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, and *Aspergillus niger* alpha-glucosidase.

The control sequence may also be a signal peptide coding region that codes for an amino acid sequence linked to the amino terminus of the heterologous polypeptide and directs

the encoded polypeptide into the cell's secretory pathway. The 5' end of the coding sequence of the nucleic acid sequence may inherently contain a signal peptide coding region naturally linked in translation reading frame with the segment of the coding region that encodes the secreted polypeptide. Alternatively, the 5' end of the coding sequence may contain a signal peptide coding region that is foreign to the coding sequence. The foreign signal peptide coding region may be required where the coding sequence does not naturally contain a signal peptide coding region. Alternatively, the foreign signal peptide coding region may simply replace the natural signal peptide coding region in order to enhance secretion of the polypeptide. The signal peptide coding region may be obtained from a glucoamylase or an amylase gene from an *Aspergillus* species, or a lipase or proteinase gene from a *Rhizomucor* species. However, any signal peptide coding region that directs the expressed heterologous polypeptide into the secretory pathway of a filamentous fungal cell may be used in the present invention.

An effective signal peptide coding region is the signal peptide coding region obtained from the genes encoding *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* neutral amylase, *Rhizomucor miehei* aspartic proteinase gene, and *Humicola lanuginosa* cellulase.

The control sequence may also be a propeptide coding region that codes for an amino acid sequence positioned at the amino terminus of a polypeptide. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally inactive and can be converted to a mature, active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding region may be obtained from the genes encoding *Rhizomucor miehei* aspartic proteinase and *Myceliophthora thermophila* laccase (WO 95/33836).

Where both signal peptide and propeptide regions are present at the amino terminus of a polypeptide, the propeptide region is positioned next to the amino terminus of the polypeptide and the signal peptide region is positioned next to the amino terminus of the propeptide region.

The nucleic acid constructs may also comprise one or more nucleic acid sequences that encode one or more factors that are advantageous for directing the expression of the heterologous polypeptide, *e.g.*, a transcriptional activator (*e.g.*, a *trans*-acting factor), chaperone, and processing protease. Any factor that is functional in a filamentous fungal cell may be used in the present invention. The nucleic acids encoding one or more of these

factors are not necessarily in tandem with the nucleic acid sequence encoding the heterologous polypeptide.

It may also be desirable to add regulatory sequences that allow the regulation of the expression of the heterologous polypeptide relative to the growth of the filamentous fungal cell. Examples of regulatory systems are those that cause the expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. The TAKA alpha-amylase promoter, *Aspergillus niger* glucoamylase promoter, and *Aspergillus oryzae* glucoamylase promoter may be used as regulatory sequences. Other examples of regulatory sequences are those that allow for gene amplification, *e.g.*, the metallothionein genes which are amplified with heavy metals. In these cases, the nucleic acid sequence encoding the heterologous polypeptide would be operably linked with the regulatory sequence.

The various nucleic acid and control sequences described above may be joined together to produce a recombinant expression vector that may include one or more convenient restriction sites to allow for insertion or substitution of the nucleic acid sequence encoding the heterologous polypeptide at such sites. Alternatively, the nucleic acid sequence encoding the heterologous polypeptide may be expressed by inserting the sequence or a nucleic acid construct comprising the sequence into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression, and possibly secretion.

The recombinant expression vector may be any vector (*e.g.*, a plasmid or virus) that can be conveniently subjected to recombinant DNA procedures and can bring about the expression of the nucleic acid sequence encoding the heterologous polypeptide. The choice of the vector will typically depend on the compatibility of the vector with the filamentous fungal cell into which the vector is to be introduced. The vector may be a linear or closed circular plasmid. The vector may be an autonomously replicating vector, *i.e.*, a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one that, when introduced into the filamentous fungal cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. The vector system may be a single vector or plasmid or two or more vectors or

plasmids that together contain the total DNA to be introduced into the genome of the filamentous fungal cell, or a transposon.

The vector preferably contains one or more selectable markers that permit easy selection of transformed filamentous fungal cells. A selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like. A selectable marker for use in a filamentous fungal host cell may be selected from the group including, but not limited to, *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *bar* (phosphinothricin acetyltransferase), *hygB* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenyltransferase), and *trpC* (anthranilate synthase), as well as equivalents thereof. Preferred for use in a filamentous fungal cell are the *amdS* and *pyrG* genes of *Aspergillus nidulans* or *Aspergillus oryzae* and the *bar* gene of *Streptomyces hygroscopicus*.

The vector preferably contains an element(s) that permits stable integration of the vector into a filamentous fungal cell genome or autonomous replication of the vector in the cell independent of the genome of the cell.

"Introduction" means introducing a vector comprising the nucleic acid sequence into a filamentous fungal cell so that the vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector. Integration is generally considered to be an advantage as the nucleic acid sequence is more likely to be stably maintained in the cell. Integration of the vector into the chromosome occurs by homologous recombination, non-homologous recombination, or transposition.

The introduction of an expression vector into a filamentous fungal cell may involve a process consisting of protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* host cells are described in EP 238 023 and Yelton *et al.*, 1984, *Proceedings of the National Academy of Sciences USA* 81: 1470-1474. A suitable method of transforming *Fusarium* species is described by Malardier *et al.*, 1989, *Gene* 78: 147-156 or in WO 96/00787.

For integration into the genome of a filamentous fungal cell, the vector may rely on the nucleic acid sequence encoding the heterologous polypeptide or any other element of the vector for stable integration of the vector into the genome by homologous or nonhomologous recombination. Alternatively, the vector may contain additional nucleic acid sequences for

directing integration by homologous recombination into the genome of the filamentous fungal cell. The additional nucleic acid sequences enable the vector to be integrated into the genome at a precise location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements should preferably contain a sufficient number of nucleic acids, such as 100 to 1,500 base pairs, preferably 400 to 1,500 base pairs, and most preferably 800 to 1,500 base pairs, which are highly homologous with the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequences that are homologous with the target sequence in the genome of the filamentous fungal cell. Furthermore, the integrational elements may be non-encoding or encoding nucleic acid sequences. On the other hand, the vector may be integrated into the genome of the cell by non-homologous recombination.

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the filamentous fungal cell in question.

It will be understood that the methods of the present invention are not limited to a particular order for obtaining the mutant filamentous fungal cell. The modification of a gene involved in the production of a cyclohexadepsipeptide may be introduced into the parent cell at any step in the construction of the cell for the production of a heterologous polypeptide. It is preferable that the filamentous fungal mutant has already been made cyclohexadepsipeptide-deficient using the methods of the present invention prior to the introduction of a gene encoding a heterologous polypeptide.

The procedures used to ligate the elements described herein to construct the recombinant expression vectors are well known to one skilled in the art (see, *e.g.*, J. Sambrook, E.F. Fritsch, and T. Maniatus, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York).

The present invention also relates to methods for obtaining cyclohexadepsipeptide-deficient filamentous fungal mutant cells which comprise (a) introducing into a parent filamentous fungal cell a first nucleic acid sequence comprising a modification of at least one of the genes involved in the production of a cyclohexadepsipeptide and a second nucleic acid sequence encoding a heterologous polypeptide; and (b) identifying the mutant from step (a) comprising the modified nucleic acid sequence, wherein the mutant cell produces less of the cyclohexadepsipeptide than the parent filamentous fungal cell of the mutant cell when cultured under the same conditions.

The present invention also relates to cyclohexadepsipeptide-deficient mutants of filamentous fungal cells for producing a heterologous polypeptide which comprise a first nucleic acid sequence comprising a modification of at least one of the genes involved in the production of a cyclohexadepsipeptide and a second nucleic acid sequence encoding the heterologous polypeptide, wherein the mutant produces less of the cyclohexadepsipeptide than the parent filamentous fungal cell of the mutant cell when cultured under the same conditions.

The present invention also relates to isolated cyclohexadepsipeptide synthetases. The term "cyclohexadepsipeptide synthetase activity" is defined herein as a synthetase activity which catalyzes the production of a cyclohexadepsipeptide from D-2-hydroxyisovaleric acid, a branched chain L-amino acid (*e.g.*, valine, leucine, isoleucine), S-adenosylmethionine, and ATP. For purposes of the present invention, cyclohexadepsipeptide synthetase activity is determined by measuring the production of a cyclohexadepsipeptide according to the procedure of Zocher *et al.*, 1982, *Biochemistry* 21: 43-48. Specifically, the cyclohexadepsipeptide synthetase is incubated with 1 mM valine, 0.2 mM S-adenosylmethionine, 0.2 mM D-2-hydroxyisovaleric acid, 4 mM ATP, and 4 mM $Mg(OAc)_2$ in a total volume of 100 µl for 10 minutes at 37°C in 50 mM MOPS pH 7.0. The amount of cyclohexadepsipeptide is determined as described herein based on the method of Visconti *et al.*, 1992, *supra*. One unit of cyclohexadepsipeptide synthetase activity is defined as 1.0 µmole of cyclohexadepsipeptide produced per minute at 37°C, pH 7.0.

In a first embodiment, the present invention relates to isolated cyclohexadepsipeptide synthetases having an amino acid sequence which has a degree of identity to the mature polypeptide contained within SEQ ID NO:2 of at least about 65%, preferably at least about 70%, more preferably at least about 80%, even more preferably at least about 90%, most preferably at least about 95%, and even most preferably at least about 97%, which have cyclohexadepsipeptide synthetase activity (hereinafter "homologous cyclohexadepsipeptide synthetases"). In a preferred embodiment, the homologous cyclohexadepsipeptide synthetases have an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from the mature polypeptide contained within SEQ ID NO:2. For purposes of the present invention, the degree of identity between two amino acid sequences is determined by the Clustal method (Higgins, 1989, *CABIOS* 5: 151-

153) using the LASERGENE™ MEGALIGN™ software (DNASTAR, Inc., Madison, WI) with an identity table and the following multiple alignment parameters: Gap penalty of 10, and gap length penalty of 10. Pairwise alignment parameters were Ktuple=1, gap penalty=3, windows=5, and diagonals=5.

Preferably, the cyclohexadepsipeptide synthetases of the present invention comprise the amino acid sequence of SEQ ID NO:2 or an allelic variant thereof; or a fragment thereof that has cyclohexadepsipeptide synthetase activity. In a more preferred embodiment, the cyclohexadepsipeptide synthetase of the present invention comprises the amino acid sequence of SEQ ID NO:2. In another preferred embodiment, the cyclohexadepsipeptide synthetase of the present invention comprises the mature polypeptide contained within SEQ ID NO:2, or an allelic variant thereof; or a fragment thereof that has cyclohexadepsipeptide synthetase activity. In another preferred embodiment, the cyclohexadepsipeptide synthetase of the present invention comprises the mature polypeptide contained within SEQ ID NO:2. In another preferred embodiment, the cyclohexadepsipeptide synthetase of the present invention consists of the amino acid sequence of SEQ ID NO:2 or an allelic variant thereof; or a fragment thereof that has cyclohexadepsipeptide synthetase activity. In another preferred embodiment, the cyclohexadepsipeptide synthetase of the present invention consists of the amino acid sequence of SEQ ID NO:2. In another preferred embodiment, the cyclohexadepsipeptide synthetase consists of the mature polypeptide contained within SEQ ID NO:2 or an allelic variant thereof; or a fragment thereof that has cyclohexadepsipeptide synthetase activity. In another preferred embodiment, the cyclohexadepsipeptide synthetase consists of the mature polypeptide contained within SEQ ID NO:2.

A fragment of SEQ ID NO:2 is a polypeptide having one or more amino acids deleted from the amino and/or carboxyl terminus of this amino acid sequence. Preferably, a fragment contains at least 2854 amino acid residues, more preferably at least 2954 amino acid residues, and most preferably at least 3054 amino acid residues.

An allelic variant denotes any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequences. An allelic variant of a polypeptide is a polypeptide encoded by an allelic variant of a gene.

The amino acid sequences of the homologous cyclohexadepsipeptide synthetases may

differ from the amino acid sequence of SEQ ID NO:2 or the mature polypeptide thereof by an insertion or deletion of one or more amino acid residues and/or the substitution of one or more amino acid residues by different amino acid residues. Preferably, amino acid changes are of a minor nature, that is conservative amino acid substitutions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the group of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions that do not generally alter the specific activity are known in the art and are described, for example, by H. Neurath and R.L. Hill, 1979, *In, The Proteins*, Academic Press, New York. The most commonly occurring exchanges are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly as well as these in reverse.

In a second embodiment, the present invention relates to isolated cyclohexadepsipeptide synthetases encoded by nucleic acid sequences that hybridize under low stringency conditions, more preferably medium stringency conditions, even more preferably high stringency conditions, and most preferably very high stringency conditions with a nucleic acid probe that hybridizes under the same conditions with (i) the nucleic acid sequence of SEQ ID NO:1, (ii) the cDNA sequence of SEQ ID NO:1, (iii) a subsequence of (i) or (ii), or (iv) a complementary strand of (i), (ii), or (iii) (J. Sambrook, E.F. Fritsch, and T. Maniatus, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York). The subsequence of SEQ ID NO:1 may be at least 100 nucleotides or preferably at least 200 nucleotides. Moreover, the subsequence may encode a polypeptide fragment that has cyclohexadepsipeptide synthetase activity. The cyclohexadepsipeptide synthetases may also be allelic variants or fragments that have cyclohexadepsipeptide synthetase activity.

The nucleic acid sequence of SEQ ID NO:1 or a subsequence thereof, as well as the

amino acid sequence of SEQ ID NO:2 or a fragment thereof, may be used to design a nucleic acid probe to identify and clone DNA encoding cyclohexadepsipeptide synthetases from strains of different genera or species according to methods well known in the art. In particular, such probes can be used for hybridization with the genomic or cDNA of the genus or species of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence, but should be at least 15, preferably at least 25, and more preferably at least 35 nucleotides in length. Longer probes can also be used. Both DNA and RNA probes can be used. The probes are typically labeled for detecting the corresponding gene (for example, with $^{32}P$, $^{3}H$, $^{35}S$, biotin, or avidin). Such probes are encompassed by the present invention.

Thus, a genomic DNA or cDNA library prepared from such other organisms may be screened for DNA that hybridizes with the probes described above and encodes a cyclohexadepsipeptide synthetase. Genomic or other DNA from such other organisms may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to and immobilized on nitrocellulose or other suitable carrier material. In order to identify a clone or DNA that is homologous with SEQ ID NO:1 or a subsequence thereof, the carrier material is used in a Southern blot. For purposes of the present invention, hybridization indicates that the nucleic acid sequence hybridizes to a nucleic acid probe corresponding to (i) the nucleic acid sequence of SEQ ID NO:1, (ii) the cDNA sequence of SEQ ID NO:1, (iii) a subsequence of (i) or (ii), or (iv) a complementary strand of (i), (ii), or (iii) under low to very high stringency conditions. Molecules to which the nucleic acid probe hybridizes under these conditions are detected using X-ray film.

In a preferred embodiment, the nucleic acid probe is a nucleic acid sequence that encodes the cyclohexadepsipeptide synthetase of SEQ ID NO:2, or a subsequence thereof. In another preferred embodiment, the nucleic acid probe is SEQ ID NO:1. In another preferred embodiment, the nucleic acid probe is the mature polypeptide coding region contained within SEQ ID NO:1. In another preferred embodiment, the nucleic acid probe is the nucleic acid sequences contained in plasmid pZL-ESA, which is contained in *Escherichia coli* NRRL B-30068, plasmid pZL-ESB, which is contained in *Escherichia coli* NRRL B-30069, and plasmid pZL-ESC, which is contained in *Escherichia coli* NRRL B-30070, wherein the nucleic acid sequences encode the cyclohexadepsipeptide synthetase of SEQ ID NO:2. In

another preferred embodiment, the nucleic acid probe is the nucleic acid sequence encoding the mature cyclohexadepsipeptide synthetase of SEQ ID NO:2 contained in plasmid pZL-ESA, which is contained in *Escherichia coli* NRRL B-30068, plasmid pZL-ESB, which is contained in *Escherichia coli* NRRL B-30069, and plasmid pZL-ESC, which is contained in *Escherichia coli* NRRL B-30070.

For long probes of at least 100 nucleotides in length, low to very high stringency conditions are defined as prehybridization and hybridization at 45°C in 5X SSPE, 0.3% SDS, 200 μg/ml sheared and denatured salmon sperm DNA, and either 25% formamide for very low and low stringencies, 35% formamide for medium and medium-high stringencies, or 50% formamide for high and very high stringencies, following standard Southern blotting procedures.

For long probes of at least 100 nucleotides in length, the carrier material is finally washed three times each for 15 minutes using 2 x SSC, 0.2% SDS preferably at least at 45°C (very low stringency), more preferably at least at 50°C (low stringency), more preferably at least at 55°C (medium stringency), more preferably at least at 60°C (medium-high stringency), even more preferably at least at 65°C (high stringency), and most preferably at least at 70°C (very high stringency).

For short probes that are about 15 nucleotides to about 70 nucleotides in length, stringency conditions are defined as prehybridization, hybridization, and washing post-hybridization at 5°C to 10°C below the calculated $T_m$ using the calculation according to Bolton and McCarthy (1962, *Proceedings of the National Academy of Sciences USA* 48:1390) in 0.9 M NaCl, 0.09 M Tris-HCl pH 7.6, 6 mM EDTA, 0.5% NP-40, 1X Denhardt's solution, 1 mM sodium pyrophosphate, 1 mM sodium monobasic phosphate, 0.1 mM ATP, and 0.2 mg of yeast RNA per ml following standard Southern blotting procedures.

For short probes that are about 15 nucleotides to about 70 nucleotides in length, the carrier material is washed once in 6X SCC plus 0.1% SDS for 15 minutes and twice each for 15 minutes using 6X SSC at 5°C to 10°C below the calculated $T_m$.

In a third embodiment, the present invention relates to isolated polypeptides having immunochemical identity or partial immunochemical identity to the cyclohexadepsipeptide synthetase having the amino acid sequence of SEQ ID NO:2 or the mature polypeptide thereof. The immunochemical properties are determined by immunological cross-reaction identity tests by the well-known Ouchterlony double immunodiffusion procedure.

Specifically, an antiserum containing polyclonal antibodies that are immunoreactive or bind to epitopes of the polypeptide having the amino acid sequence of SEQ ID NO:2 or the mature polypeptide thereof are prepared by immunizing rabbits (or other rodents) according to the procedure described by Harboe and Ingild, *In* N.H. Axelsen, J. Krøll, and B. Weeks, editors, *A Manual of Quantitative Immunoelectrophoresis*, Blackwell Scientific Publications, 1973, Chapter 23, or Johnstone and Thorpe, *Immunochemistry in Practice*, Blackwell Scientific Publications, 1982 (more specifically pages 27-31). A polypeptide having immunochemical identity is a polypeptide that reacts with the antiserum in an identical fashion such as total fusion of precipitates, identical precipitate morphology, and/or identical electrophoretic mobility using a specific immunochemical technique. A further explanation of immunochemical identity is described by Axelsen, Bock, and Krøll, *In* N.H. Axelsen, J. Krøll, and B. Weeks, editors, *A Manual of Quantitative Immunoelectrophoresis*, Blackwell Scientific Publications, 1973, Chapter 10. A polypeptide having partial immunochemical identity is a polypeptide that reacts with the antiserum in a partially identical fashion such as partial fusion of precipitates, partially identical precipitate morphology, and/or partially identical electrophoretic mobility using a specific immunochemical technique. A further explanation of partial immunochemical identity is described by Bock and Axelsen, *In* N.H. Axelsen, J. Krøll, and B. Weeks, editors, *A Manual of Quantitative Immunoelectrophoresis*, Blackwell Scientific Publications, 1973, Chapter 11.

The antibody may also be a monoclonal antibody. Monoclonal antibodies may be prepared and used, *e.g.*, according to the methods of E. Harlow and D. Lane, editors, 1988, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, New York.

The isolated cyclohexadepsipeptide synthetases of the present invention have at least 20%, preferably at least 40%, more preferably at least 60%, even more preferably at least 80%, even more preferably at least 90%, and most preferably at least 100% of the cyclohexadepsipeptide synthetase activity of the mature polypeptide of SEQ ID NO:2.

In a preferred embodiment, a cyclohexadepsipeptide synthetase of the present invention is obtained from a *Fusarium venenatum* strain, and more preferably from *Fusarium venenatum* ATCC 20334 or a mutant strain thereof, *e.g.*, the polypeptide with the amino acid sequence of SEQ ID NO:2.

As defined herein, an "isolated" cyclohexadepsipeptide synthetase is a polypeptide

that is essentially free of other polypeptides, *e.g.*, at least about 20% pure, preferably at least about 40% pure, more preferably about 60% pure, even more preferably about 80% pure, most preferably about 90% pure, and even most preferably about 95% pure, as determined by SDS-PAGE.

A cyclohexadepsipeptide synthetase of the present invention may be obtained from microorganisms of any genus.

A cyclohexadepsipeptide synthetase of the present invention may be a bacterial cyclohexadepsipeptide synthetase. For example, the cyclohexadepsipeptide synthetase may be a gram positive bacterial cyclohexadepsipeptide synthetase such as a *Bacillus* cyclohexadepsipeptide synthetase, *e.g.*, a *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus coagulans*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus stearothermophilus*, *Bacillus subtilis*, or *Bacillus thuringiensis* cyclohexadepsipeptide synthetase; or a *Streptomyces* cyclohexadepsipeptide synthetase, *e.g.*, a *Streptomyces lividans* or *Streptomyces murinus* cyclohexadepsipeptide synthetase; or a gram negative bacterial cyclohexadepsipeptide synthetase, *e.g.*, an *E. coli* or a *Pseudomonas* sp. cyclohexadepsipeptide synthetase.

A cyclohexadepsipeptide synthetase of the present invention may be a fungal cyclohexadepsipeptide synthetase, and more preferably a yeast cyclohexadepsipeptide synthetase such as a *Candida*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* cyclohexadepsipeptide synthetase; or more preferably a filamentous fungal cyclohexadepsipeptide synthetase such as an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Piromyces*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, or *Trichoderma* cyclohexadepsipeptide synthetase.

In a preferred embodiment, the cyclohexadepsipeptide synthetase is a *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis* or *Saccharomyces oviformis* cyclohexadepsipeptide synthetase.

In another preferred embodiment, the cyclohexadepsipeptide synthetase is an *Aspergillus aculeatus*, *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Humicola insolens*, *Humicola*

*lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cyclohexadepsipeptide synthetase.

In another preferred embodiment, the cyclohexadepsipeptide synthetase is a *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, or *Fusarium venenatum* cyclohexadepsipeptide synthetase.

The present invention also relates to isolated nucleic acid sequences that encode a cyclohexadepsipeptide synthetase of the present invention. In a preferred embodiment, the nucleic acid sequence is set forth in SEQ ID NO:1. In another more preferred embodiment, the nucleic acid sequence is the sequences contained in plasmid pZL-ESA, which is contained in *Escherichia coli* NRRL B-30068, plasmid pZL-ESB, which is contained in *Escherichia coli* NRRL B-30069, and plasmid pZL-ESC, which is contained in *Escherichia coli* NRRL B-30070. In another more preferred embodiment, the nucleic acid sequence is the sequences encoding the mature polypeptide contained within SEQ ID NO:2 that is contained in plasmid pZL-ESA, which is contained in *Escherichia coli* NRRL B-30068, plasmid pZL-ESB, which is contained in *Escherichia coli* NRRL B-30069, and plasmid pZL-ESC, which is contained in *Escherichia coli* NRRL B-30070. In another preferred embodiment, the nucleic acid sequence is the mature polypeptide coding region contained within SEQ ID NO:1. The present invention also encompasses nucleic acid sequences that encode a polypeptide having the amino acid sequence of SEQ ID NO:2 or the mature polypeptide thereof, which differ from SEQ ID NO:1 by virtue of the degeneracy of the genetic code. The present invention also relates to subsequences of SEQ ID NO:1 that encode fragments of SEQ ID NO:2, which have cyclohexadepsipeptide synthetase activity.

A subsequence of SEQ ID NO:1 is a nucleic acid sequence encompassed by SEQ ID NO:1 except that one or more nucleotides from the 5' and/or 3' end have been deleted. Preferably, a subsequence contains at least 8562 nucleotides, more preferably at least 8862 nucleotides, and most preferably at least 9162 nucleotides.

The present invention also relates to mutant nucleic acid sequences comprising at least

one mutation in the mature polypeptide coding sequence of SEQ ID NO:1, in which the mutant nucleic acid sequence encodes a polypeptide which consists of the mature polypeptide contained within SEQ ID NO:2.

The techniques used to isolate or clone a nucleic acid sequence encoding a cyclohexadepsipeptide synthetase may include isolation from genomic DNA, preparation from cDNA, or a combination thereof, as described herein. The cloning of the nucleic acid sequences of the present invention from such genomic DNA can be effected, *e.g.*, by PCR or other nucleic acid amplification procedures such as ligase chain reaction (LCR), ligated activated transcription (LAT) and nucleic acid sequence-based amplification (NASBA) may be used. The nucleic acid sequence may be cloned from a strain of *Fusarium*, or another or related organism and thus, for example, may be an allelic or species variant of the polypeptide encoding region of the nucleic acid sequence. The nucleic acid sequence may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

The term "isolated nucleic acid sequence" as used herein refers to a nucleic acid sequence that is essentially free of other nucleic acid sequences, *e.g.*, at least about 20% pure, preferably at least about 40% pure, more preferably at least about 60% pure, even more preferably at least about 80% pure, and most preferably at least about 90% pure as determined by agarose electrophoresis.

The present invention also relates to nucleic acid sequences that have a degree of homology to the mature polypeptide coding region contained within SEQ ID NO:1 of at least about 65%, preferably about 70%, preferably about 80%, more preferably about 90%, even more preferably about 95%, and most preferably about 97% homology, which encode an active polypeptide. For purposes of the present invention, the degree of homology between two nucleic acid sequences is determined by the Wilbur-Lipman method (Wilbur and Lipman, 1983, *Proceedings of the National Academy of Science USA* 80: 726-730) using the LASERGENE™ MEGALIGN™ software (DNASTAR, Inc., Madison, WI) with an identity table and the following multiple alignment parameters: Gap penalty of 10, and gap length penalty of 10. Pairwise alignment parameters were Ktuple=3, gap penalty=3, and windows=20.

Modification of a nucleic acid sequence encoding a cyclohexadepsipeptide synthetase of the present invention may be necessary for the synthesis of polypeptides substantially similar to the cyclohexadepsipeptide synthetase. The term "substantially similar" to the

cyclohexadepsipeptide synthetase refers to non-naturally occurring forms of the enzyme. These polypeptides may differ in some engineered way from the cyclohexadepsipeptide synthetase isolated from its native source, *e.g.*, variants of the cyclohexadepsipeptide synthetase that differ in specific activity, thermostability, pH optimum, or the like. The variant sequence may be constructed on the basis of the nucleic acid sequence presented as the polypeptide encoding part of SEQ ID NO:1, *e.g.*, a subsequence thereof, and/or by introduction of nucleotide substitutions that do not give rise to another amino acid sequence of the polypeptide encoded by the nucleic acid sequence, but which correspond to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions that may give rise to a different amino acid sequence. For a general description of nucleotide substitution, see, *e.g.*, Ford *et al.*, 1991, *Protein Expression and Purification* 2: 95-107.

It will be apparent to those skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active polypeptide. Amino acid residues essential to the activity of the cyclohexadepsipeptide synthetase encoded by the isolated nucleic acid sequence of the invention, and therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (see, *e.g.*, Cunningham and Wells, 1989, *Science* 244: 1081-1085). In the latter technique, mutations are introduced at every positively charged residue in the molecule, and the resultant mutant molecules are tested for cyclohexadepsipeptide synthetase activity to identify amino acid residues that are critical to the activity of the molecule. Sites of substrate-enzyme interaction can also be determined by analysis of the three-dimensional structure as determined by such techniques as nuclear magnetic resonance analysis, crystallography or photoaffinity labelling (see, *e.g.*, de Vos *et al.*, 1992, *Science* 255: 306-312; Smith *et al.*, 1992, *Journal of Molecular Biology* 224: 899-904; Wlodaver *et al.*, 1992, *FEBS Letters* 309: 59-64).

The present invention also relates to isolated nucleic acid sequences encoding a cyclohexadepsipeptide synthetase of the present invention, which hybridize under low stringency conditions, more preferably medium stringency conditions, even more preferably high stringency conditions, and most preferably very high stringency conditions as defined herein with a nucleic acid probe that hybridizes under the same conditions with (i) the nucleic acid sequence of SEQ ID NO:1, (ii) the cDNA sequence of SEQ ID NO:1, (iii) a subsequence

of (i) or (ii), or (iv) a complementary strand of (i), (ii), or (iii); or allelic variants thereof (Sambrook *et al.*, 1989, *supra*), as defined herein.

The present invention also relates to isolated nucleic acid sequences produced by (a) hybridizing a DNA under low, medium, high, or very high stringency conditions with (i) the nucleic acid sequence of SEQ ID NO:1, (ii) the cDNA sequence of SEQ ID NO:1, (iii) a subsequence of (i) or (ii), or (iv) a complementary strand of (i), (ii), or (iii); and (b) isolating the nucleic acid sequence. The subsequence is preferably a sequence of at least 100 nucleotides such as a sequence that encodes a polypeptide fragment, which has cyclohexadepsipeptide synthetase activity.

The present invention further relates to methods for producing a mutant nucleic acid sequence, comprising introducing at least one mutation into the mature polypeptide coding sequence of SEQ ID NO:1 or a subsequence thereof, wherein the mutant nucleic acid sequence encodes a polypeptide which consists of the mature polypeptide contained within SEQ ID NO:2 or a fragment thereof that has cyclohexadepsipeptide synthetase activity.

The introduction of a mutation into the nucleic acid sequence to exchange one nucleotide for another nucleotide may be accomplished by site-directed mutagenesis using any of the methods known in the art. Particularly useful is the procedure which utilizes a supercoiled, double stranded DNA vector with an insert of interest and two synthetic primers containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, extend during temperature cycling by means of *Pfu* DNA polymerase. On incorporation of the primers, a mutated plasmid containing staggered nicks is generated. Following temperature cycling, the product is treated with *Dpn*I which is specific for methylated and hemimethylated DNA to digest the parental DNA template and to select for mutation-containing synthesized DNA. Other procedures known in the art may also be used.

The present invention also relates to nucleic acid constructs, recombinant expression vectors, and host cells containing the nucleic acid sequence of SEQ ID NO:1, subsequences or homologues thereof, for expression of the sequences. The constructs and vectors may be constructed as described herein. The host cell may be any cell suitable for the expression of the nucleic acid sequence.

The host cell may be a eukaryote, such as a mammalian cell, an insect cell, a plant cell or a fungal cell. Useful mammalian cells include Chinese hamster ovary (CHO) cells, HeLa

cells, baby hamster kidney (BHK) cells, COS cells, or any number of other immortalized cell lines available, *e.g.*, from the American Type Culture Collection.

In a preferred embodiment, the host cell is a fungal cell. In a more preferred embodiment, the fungal host cell is a yeast cell or a filamentous fungal cell.

In an even more preferred embodiment, the yeast host cell is a *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* cell.

In a most preferred embodiment, the yeast host cell is a *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis* or *Saccharomyces oviformis* cell. In another most preferred embodiment, the yeast host cell is a *Kluyveromyces lactis* cell. In another most preferred embodiment, the yeast host cell is a *Yarrowia lipolytica* cell.

In another even more preferred embodiment, the filamentous fungal host cell is an *Acremonium*, *Aspergillus*, *Fusarium*, *Humicola*, *Mucor*, *Myceliophthora*, *Neurospora*, *Penicillium*, *Thielavia*, *Tolypocladium*, or *Trichoderma* cell.

In a most preferred embodiment, the filamentous fungal host cell is an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger* *Aspergillus oryzae*, *Fusarium bactridioides*, *Fusarium crookwellense* (synonym of *Fusarium cerealis*), *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium solani*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, (*e.g.*, *Fusarium venenatum* (Nirenberg sp. nov.), *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophilum*, *Neurospora crassa*, *Penicillium purpurogenum*, *Thielavia terrestris*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cell.

Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* and *Fusarium* host cells are described herein. Yeast may be transformed using the procedures described by Becker and Guarente, *In* Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology*, Methods in Enzymology, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito *et al.*, 1983, *Journal of Bacteriology* 153: 163; and Hinnen *et al.*, 1978, *Proceedings of the*

*National Academy of Sciences USA* 75: 1920.

The present invention also relates to methods for producing a cyclohexadepsipeptide synthetase of the present invention comprising (a) cultivating a strain, which in its wild-type form is capable of producing the cyclohexadepsipeptide synthetase, to produce a supernatant comprising the cyclohexadepsipeptide synthetase; and (b) recovering the cyclohexadepsipeptide synthetase. Preferably, the strain is of the genus *Fusarium*, and more preferably *Fusarium venenatum*.

The present invention also relates to methods for producing a cyclohexadepsipeptide synthetase of the present invention comprising (a) cultivating a host cell under conditions conducive for production of the cyclohexadepsipeptide synthetase; and (b) recovering the cyclohexadepsipeptide synthetase.

In the production methods of the present invention, the cells are cultivated in a nutrient medium suitable for production of the cyclohexadepsipeptide synthetase using methods known in the art as described herein. The cyclohexadepsipeptide synthetase may be detected using methods known in the art specific for the enzyme (see, *e.g.*, Visconti *et al.*, 1992, *supra*). The resulting cyclohexadepsipeptide synthetase may be recovered and purified by methods known in the art as described herein.

The present invention also relates to methods for producing cyclohexadepsipeptides and to cyclohexadepsipeptides produced by the cyclohexadepsipeptide synthetases of the present invention. The production of a cyclohexadepsipeptide may be accomplished with the isolated synthetase or by fermentation of a cell containing the gene encoding the synthetase (see, for example, Madry *et al.*, 1983, *European Journal of Applied Microbiology and Biotechnology* 17: 75-79).. The cell may be a wild-type cell or a recombinant cell. The cyclohexadepsipeptides may be isolated and purified by any of the methods known in the art. See, for example, U.S. Patent No. 5,656,464; Visconti *et al.*, 1992, *supra*.

In a preferred embodiment, the method for producing a cyclohexadepsipeptide, comprises: (a) reacting a cyclohexadepsipeptide synthetase of the present invention with D-2-hydroxyisovaleric acid, a branched chain L-amino acid, S-adenosylmethionine, and ATP; and (b) isolating the cyclohexadepsipeptide from the reaction.

In another preferred embodiment, the method for producing a cyclohexadepsipeptide, comprises: (a) cultivating a cell under conditions suitable for the production of the cyclohexadepsipeptide, wherein the cell comprises a nucleic acid sequence encoding (i) a

cyclohexadepsipeptide synthetase having an amino acid sequence which has at least 65% identity with the mature polypeptide contained within SEQ ID NO:2; (ii) a cyclohexadepsipeptide synthetase which is encoded by a nucleic acid sequence which hybridizes under medium stringency conditions with the nucleic acid sequence of SEQ ID NO:1 or its complementary strand, or a subsequence of SEQ ID NO:1 of at least 100 nucleotides; (iii) an allelic variant of (a) or (b); or (iv) a fragment of (a), (b), or (c) that has cyclohexadepsipeptide synthetase activity; and (b) isolating the cyclohexadepsipeptide from the reaction.

The present invention is further described by the following examples which should not be construed as limiting the scope of the invention.

## Examples

### Strains

*Fusarium venenatum* strain ATCC 20334 was used as the source of genomic DNA for these experiments. Genomic DNA libraries were constructed using the λZipLox cloning system (Life Technologies, Gaithersburg, MD) with *E. coli* Y1090ZL as a host for plating and purification of recombinant bacteriophage and *E. coli* DH10Bzip for excision of recombinant pZL1-derivatives. *Fusarium torulosum* R-5690 (Fusarium Research Center, Penn State University, State College, PA) and *Aspergillus niger* Bo-1 (Novo Nordisk A/S, Bagsvaerd, Denmark) were used as sources of control DNAs for hybridization experiments. The *tri5*-deleted *Fusarium venenatum* strain LyMC1A (WO 99/60137) was used as the recipient for transformation experiments. *Escherichia coli* TOP10 (Invitrogen Corp., Carlsbad, CA) and *E. coli* DH5-alpha strains (Gibco-BRL Life Technologies, Bethesda, MD) were used for vector construction and routine plasmid propagation.

### Media

RA sporulation medium was composed per liter of 50 g of succinic acid (disodium salt), 20 ml of 50X Vogels salts, 12.1 g of $NaNO_3$, and 1 g of glucose.

50X Vogels Salts was composed per liter of 125 g of sodium citrate, 250 g of $KH_2PO_4$, 10 g of $MgSO_4 \cdot 7H_20$, 5 g of $CaCl_2 \cdot 2H_20$ (predissolved in 20 ml water), and 5 ml of 200X Vogels trace elements. (Each ingredient was dissolved completely before addition of

the next one). Filter sterilized.

200 X Vogels Trace Elements was composed per 100 ml of 5g of citric acid·$1H_20$, 5 g of $ZnSO_4 \cdot 7H_20$, 1 g of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_20$, 0.25 g of $CuSO_4 \cdot 5H_20$, 0.05 g of $MnSO_4 \cdot 1H_20$, 0.05 g of $H_3BO_3$, and 0.05 g of $Na_2MoO_4 \cdot 2H_20$.

Fluoroacetamide agar (FA) was composed per liter of 12g of sodium acetate, 2 g of sodium chloride, 0.5 g of $MgSO_4$, 3 g of $KH_2PO_4$, 0.3 g of urea, 2 g of fluoroacetamide, 1 ml of Vogels salts, and 15 g of Noble agar (pH 6.1).

Cove medium was composed per liter of 342.3 g of sucrose, 20 ml of 50X Cove salt solution, 10 ml of 1 M acetamide, 10 ml of 1.5 M CsCl, and 25 g of Noble agar.

50X Cove Salts was composed per liter of 26g of KCl, 26 g of $MgSO_4 \cdot 7H_2O$, 76g of $KH_2PO_4$, and 50 ml of 20X Cove trace elements.

20X Cove trace elements was composed per liter of 0.04 g of $Na_2B_4O_7 \cdot 10H_2O$, 0.4 g of $CuSO_4 \cdot 5H_2O$, 1.2 g of $FeSO_4 \cdot 7H_2O$, 0.7 g of $MnSO_4 \cdot H_2O$, 0.8 g of $Na_2MoO_2 \cdot 2H_2O$, and 10 g of $ZnSO_4 \cdot 7H_2O$.

**Example 1: Genomic DNA Extraction of *Fusarium venenatum, Fusarium torulosum,* and *Aspergillus niger***

*Fusarium venenatum, Fusarium torulosum,* and *Aspergillus niger* were each grown for 24-36 hours at 28°C and 150 rpm in 25 ml of YEG medium composed per liter of 5 g of yeast extract and 20 g of glucose. Mycelia were then collected by filtration through Miracloth (Calbiochem, La Jolla, CA) and washed once with 25 ml of 10 mM Tris-1 mM EDTA (TE) buffer. Excess buffer was drained from the mycelia which were subsequently frozen in liquid nitrogen. The frozen mycelia were ground to a fine powder in an electric coffee grinder, and each powder was added to 20 ml of TE buffer and 5 ml of 20% w/v sodium dodecylsulfate (SDS) in a disposable plastic centrifuge tube. The mixtures were gently inverted several times to ensure mixing, and extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v). Sodium acetate (3 M solution) was added to give a final concentration of 0.3 M and the nucleic acids were precipitated with 2.5 volumes of ice cold ethanol. The tubes were centrifuged at 15,000 x *g* for 30 minutes and the pellets were allowed to air dry for 30 minutes before resuspension in 0.5 ml of TE buffer. DNase-free ribonuclease A was added to a concentration of 100 μg/ml and the mixtures were incubated at 37°C for 30 minutes. Proteinase K (200 μg/ml) was then added and the mixtures

were incubated an additional hour at 37°C. Finally, the mixtures were extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) before precipitating the DNA with sodium acetate and ethanol according to standard procedures. The DNA pellets were dried under vacuum, resuspended in TE buffer, and stored at 4°C.

**Example 2: Hybridization Experiments**

The genomic DNA preparations described in Example 1 were tested for the presence of cyclohexadepsipeptide synthetase gene sequences using Southern hybridization. Aliquots of the DNA were digested with *Bam*HI or *Bam*HI plus *Xba*I and fractionated by agarose gel electrophoresis. The DNA in the gel was blotted to a Hybond N+™ membrane filter (Amersham Corporation, Arlington Heights, IL) according to the method of Davis *et al.* (1980, *Advanced Bacterial Genetics, A Manual for Genetic Engineering*, Cold Spring Harbor Press, Cold Spring Harbor, NY), and probed with a radiolabeled fragment encoding the 5' portion of the *Fusarium torulosum esyn1* gene (obtained from Dr. Thomas Hohn, USDA, Peoria, IL) under low, medium, and high stringency hybridization conditions at 45°C as described herein. The cyclohexadepsipeptide synthetase-specific probe fragment from *Fusarium torulosum* was radiolabeled by nick translation (Sambrook *et al.*, 1989, *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY) with $\alpha[^{32}P]$dCTP (Amersham, Arlington Heights, IL), denatured by adding NaOH to a final concentration of 0.1 M, and added to the hybridization buffer at an activity of approximately 1 x $10^6$ cpm per ml. Following the hybridization, the filters were washed once in 0.2X SSPE with 0.1% SDS at 45°C followed by two washes in 0.2X SSPE (no SDS) at the same temperature. The filters were allowed to dry on paper towels for 15 minutes, then wrapped in Saran-wrap™ and exposed to X-ray film overnight at -70°C with intensifying screens (Kodak, Rochester, NY).

Southern hybridization analysis showed that cyclohexadepsipeptide synthetase-specific DNA sequences could be detected in the genome of *Fusarium venenatum* with the *Fusarium torulosum esyn1* probe only under conditions of low and medium stringency. The positive control DNA from *Fusarium torulosum* gave strong hybridization signals under all conditions, and negative control DNA from *Aspergillus niger* failed to hybridize under all conditions tested. These results suggested that *Fusarium venenatum* contained genomic DNA sequences homologous to the *Fusarium torulosum* enniatin synthetase gene.

**Example 3: Genomic DNA Library Construction and Screening**

Genomic libraries of *Fusarium venenatum* were constructed in λZipLox according to the manufacturer's instructions (Life Technologies, Gaithersburg, MD). *Fusarium venenatum* genomic DNA was partially digested with *Tsp*509I and size-fractionated on 1% agarose gels. DNA fragments migrating in the size range 3-7 kb were excised and eluted from the agarose gel slices using Prep-a-Gene reagents (BioRad, Hercules, CA). The eluted DNA fragments were ligated with *Eco*RI-cleaved and dephosphorylated λZipLox vector arms (Life Technologies, Gaithersburg, MD), and the ligation mixtures were packaged using commercial packaging extracts (Stratagene, La Jolla, CA). The packaged DNA libraries were plated and amplified in *E. coli* Y1090ZL cells.

Approximately 50,000 plaques from the library were screened by plaque-hybridization (Davis *et al.*, 1980, *supra*) with the radiolabeled probe fragment of the *Fusarium torulosum esyn1* gene using the low stringency conditions described in Example 2. Plaques, which gave hybridization signals, were purified twice in *E. coli* Y1090ZL cells, and the individual clones were subsequently excised from the λZipLox vector as pZL1-derivatives (D'Alessio *et al.*, 1992, *Focus*® 14: 7). Chromosome "walking" to obtain adjacent DNA sequences was done using homologous *Fusarium venenatum* probes at high stringency.

Four plaques were identified that hybridized strongly to the *Fusarium torulosum esyn1* gene probe, and each of the potential clones was subsequently excised from the λZipLox vector as a pZL1-derivative (D'Alessio *et al.*, 1992, *supra*). Plasmid DNA was isolated from the clones by passage through *E. coli* DH10B cells using standard methods. The sizes of the cloned inserts were determined by agarose gel electrophoresis. The largest insert comprised a DNA segment of approximately 3 kb. The clone was designated *E. coli* DH10B pZL-ESA.

**Example 4: Cloning and Analysis of a *Fusarium venenatum* Cyclohexadepsipeptide Synthetase Gene**

DNA sequencing of the DNA segment of approximately 3 kb was performed with an Applied Biosystems Model 377 XL Automated DNA Sequencer using dye-terminator chemistry. Contiguous sequences were generated using a transposon insertion strategy

(Primer Island Transposition Kit, Perkin-Elmer/Applied Biosystems, Inc., Foster City, CA). The entire cloned region was sequenced to an average redundancy of 6.9.

Nucleotide sequencing revealed that the 3 kb segment contained an open reading frame encoding at least 900 amino acids. However, this fragment (designated Fragment A, pZL-ESA) did not encode the entire gene product. Consequently, the library was re-screened using a probe comprising the 3'-portion of Fragment A (ca. 1 kb *Hind*III fragment). Several clones were subsequently identified and analyzed by restriction mapping. The largest of these secondary clones contained a genomic DNA insert of about 4.6 kb (designated Fragment B, pZL-ESB). The clone was designated *E. coli* DH10B pZL-ESB.

Nucleotide sequence examination of Fragment B extended the open reading frame of Fragment A by amino acids 777 through 2311. However, this sequence did not reach the stop codon of the open reading frame, thereby necessitating isolation of a third genomic segment. The third genomic clone was isolated by re-screening the genomic library with a PCR-amplified probe derived from Fragment B. Two PCR primers shown below were used to amplify a 586 bp probe segment used for screening the library.

5'-dAATTGATTCGCTTGAAAGTCGAT-3' (SEQ ID NO:3)

5'-dCTTGAGAGTTACGTTGGTCTTGAAC-3' (SEQ ID NO:4)

The amplification reaction (100 µl) contained the following components: 0.2 µg of pZL-ESB DNA, 48.4 pmol of the forward primer, 48.4 pmol of the reverse primer, 1 mM each of dATP, dCTP, dGTP, and dTTP, 1 x *Taq* polymerase buffer, and 2.5 U of *Taq* polymerase (Perkin-Elmer Corp., Branchburg, NJ). The reaction was incubated in an Ericomp Twin Block System Easy Cycler programmed for 1 cycle at 95°C for 5 minutes followed by 30 cycles each at 95°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes.

The reaction was electrophoresed on an agarose gel, and the expected product of 586 bp was obtained. The reaction was run on a preparative gel, a gel slice containing the desired product was excised, and DNA was isolated from the gel using a Qiaquick Gel Extraction Kit (Qiagen, Chatsworth, CA).

From seven clones that were identified with this probe, the largest (Fragment C, pZL-ESC) contained a 5.5 kb insert. Subsequent DNA sequence analysis revealed that Fragment C encoded amino acids 1617 through 3129, a potential stop codon, and 1553 bp of 3'-flanking DNA. The clone was designated *E. coli* DH10B pZL-ESC. The entire DNA sequence of the cyclohexadepsipeptide synthetase gene was assembled from the three

overlapping clones (Fragments A, B, and C). A transposon insertion strategy allowed for rapid sequencing to high redundancy.

The complete DNA sequence and deduced amino acid sequence are shown in Figure 1. The DNA sequence of the cyclohexadepsipeptide synthetase gene (SEQ ID NO:1) was determined to an average redundancy of 6.9. The cyclohexadepsipeptide synthetase gene contained a lengthy open reading frame of 9387 bp with no introns, encoding a polypeptide of 3129 amino acids (MW = 346,852).

The deduced amino acid sequence (SEQ ID NO:2) of the cyclohexadepsipeptide synthetase gene product shared approximately 59% identity to the enniatin synthetase of *Fusarium scirpi* (Haese *et al.*, 1993, *Mol. Microbiol.* 7: 905-914; DNA sequence listed in EMBL database under accession number Z18755). Percent identity was determined by the Clustal method (Higgins, 1989, *CABIOS* 5: 151-153) using the LASERGENE™ MEGALIGN™ software (DNASTAR, Inc., Madison, WI) with an identity table and the following multiple alignment parameters: Gap penalty of 10 and gap length penalty of 10. Pairwise alignment parameters were Ktuple=1, gap penalty=3, windows=5, and diagonals=5.

**Example 5: pΔES-*amdS* Construction**

The construction of the *dps1* deletion vectors pΔES-*amdS*1 and pΔES-*amdS*2 is shown in Figure 2. Briefly, a 0.2 kb DNA segment comprising a portion of the *dps1* coding region was removed from plasmid pZL-ESA (designated as fragment A) by digestion with *Stu*I and *Nru*I restriction endonucleases. Both of these enzymes generate blunt-ended DNA fragments. The digested pESA vector was treated with calf intestine alkaline phosphatase (Boehringer-Mannheim Biochemicals, Indianapolis, IN) to prevent self-ligation. Lastly, a 3.2 kb fragment encoding the *Aspergillus nidulans amdS* gene (with flanking repeat sequences derived from the *Aspergillus oryzae pyrG* gene) was obtained by digestion of pJRoy47 (WO 99/60137) with *Sma*I and *Pme*I. This *amdS* fragment was subsequently ligated with the pZL-ESA vector fragment described above to generate the deletion plasmids pΔES-*amdS*1 and pΔES-*amdS*2 (which differ only in the orientation of the *amdS* gene segment).

**Example 6: Transformation of *Fusarium venenatum* LyMC1A and Preliminary Screening for dps1 Gene Deletions**

Plasmid pΔES-*amdS*1 was digested with *Spe*I, and the 5.7 kb deletion fragment

(comprising portions of the *Fusarium venenatum dps1* gene with the *Aspergillus nidulans amdS* gene and repeats replacing 0.2 kb of the *dps1* coding region) was subsequently excised and purified for use in transformation experiments. The preparation and transformation of *Fusarium venenatum* LyMC1A protoplasts was performed according to the method of Royer, 1995, *Bio/Technology* 13: 1479-1483.

*Fusarium venenatum* LyMC1A protoplasts were transformed with the 5.7 kb *Spe*I ΔES-*amdS* fragment with selection on COVE plates. Fifteen transformants were obtained and single spore purified. DNA was extracted from the single spore purified transformants, generated with the *Spe*I ΔES-*amdS* fragment, as well as from *Fusarium venenatum* LyMC1A, using the Qiagen DNeasy Plant mini kit (Qiagen, Chatsworth, CA) (with a 2 hour lytic incubation in place of 10 minutes recommended in the manufacturer's protocol). One to two micrograms of each DNA were digested for seven hours with *XhoI* or *Spe*I (10 U/μg DNA in 30 μl reactions). The digests were electrophoresed on 1% agarose gels in TAE buffer, and the DNAs were transferred to Hybond $N^+$ in 0.4 N NaOH. The blots were UV crosslinked and probed as described below.

Probes were prepared using the Prime-It Labeling Kit (Stratagene, La Jolla, CA) and α[$^{32}$P]-dCTP. Following labeling the probes were separated from unincorporated label using a G 50 TE Midi column (5' to 3', Boulder, CO).

Blots were prehybridized at 65°C in Rapid Hyb Buffer (Amersham, Arlington Heights, IL) for 45 minutes. Denatured probes were added to the Rapid Hyb solution and hybridizations were done overnight at 65°C. Following hybridization the blots were washed once at room temperature in 2X SSC for 5 minutes and in 0.2X SSC, 0.1% SDS at 65°C for 5 minutes twice. The washed blots were washed in 2X SSC at room temperature for 5 minutes.

Southern blots of *Xho*I and *Spe*I-digested genomic DNA were probed twice. First, they were probed with an 800 bp *Nsi*I/*Spe*I fragment of pΔES-*amdS*1. Four of the fifteen transformants had the 5.2 kb band (*Xho*I digested DNA) and the 5.7 kb band (*Spe*I digested DNA) expected for a gene replacement when probed with the 800 bp *Nsi*I/*Spe*I fragment. Most of the other transformants had the 2.2 kb band (*Xho*I digested DNA) or the 2.7 kb (*Spe*I digested DNA) wild-type bands, and additional bands, most likely corresponding to ectopic integration of the transforming DNA.

Secondly, the same Southern blots were probed with *Hind*III-linearized pDSY176, a plasmid containing the 0.2 kb *Stu*I/*Nru*I portion of the *dps1* coding region. pDSY176 was

constructed as follows: pZL-ESA was digested with *StuI*/*NruI*, and the 0.2 kb fragment was isolated by preparative electrophoresis. The isolated fragment was cloned into pZERO-Blunt (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions to produce pDSY176. Hybridization analysis using this second probe (pDSY176) confirmed that none of the four putative deleted strains (*Fusarium venenatum* ΔES 4, 6, 8, and 10) contained the 0.2 kb region of the *dps1* gene which had been deleted.

**Example 7: Removal of the *amdS* Gene**

Two of the transformants confirmed as being deleted, ΔES4A and ΔES8A, were sporulated in 500 ml of RA medium, in 2 liter Fernbach flasks. These were inoculated with 12 mycelial plugs cut from Cove plates and incubated at 24°C, 150 rpm for 53 hours. After this time spores were harvested through sterile Miracloth (Calbiochem, San Diego, CA) centrifuged for 30 minutes at 7,000 rpm in a Sorval GS3 rotor and washed three times with sterile water. Freshly harvested spores were plated at $10^4$, $10^5$ and $10^6$ per plate (five plates at each concentration) of FA. Colonies which grew on these plates were picked to FA and Cove plates.

Numerous colonies were obtained on FA plates (primarily from plates seeded with $10^5$ and $10^6$ spores/plate) which, on subculturing, grew well on FA but only sparsely on Cove. Thirty two ΔES4A-derived colonies (designated as *Fusarium venenatum* WTY700-3-4) and 128 ΔES8A-derived colonies (designated as *Fusarium venenatum* WTY700-3-8) all had an *amdS*-minus phenotype.

DNA was extracted from five *Fusarium venenatum* WTY700-3-4 isolates (WTY700-3-4a through 4e) and ten *Fusarium venenatum* WTY700-3-8 strains (WTY700-3-8a through 8j) as well as LyMC1A, ΔES4A and ΔES8A using the Qiagen DNeasy Plant Mini Kit (with a 2 hour lytic incubation in place of 10 min recommended in the manufacturer's protocol). One microgram of each DNA was digested overnight with *XhoI* and *SpeI* (20 U/μg DNA in 50 μl reactions). The digests were concentrated to 10 μl and run on 1% agarose gels in TBE. DNAs were transferred to Hybond N+ membranes using the manufacturer's protocol, and were then probed with the probes described in Example 6.

Hybridization analysis of genomic DNAs extracted from these strains (using the 800 bp *NsiI*-*SpeI* fragment of ΔES-*amdS1* and *Hind*III-digested pDSY176 (which contains the 200 bp *StuI*-*NruI* portion of the *dps1* open reading frame) revealed that none of the WTY700-

3-4 isolates had lost the *amdS* gene, but 50% (5/10) of the *Fusarium venenatum* WTY700-3-8 isolates showed a banding pattern consistent with removal of the *amdS* gene. Hybridization analysis, thus, confirmed that *Fusarium venenatum* WTY700-3-8a, b, c, d, and e were deleted for the 200 bp *StuI*/*NruI* portion of the *dpsI* open reading frame. As expected for a deletion, a hybridization signal was observed using the pDSY176 probe described above for the parent strain *Fusarium venenatum* LyMC1A but not for any of the five *Fusarium venenatum* WTY700-3-8a-e strains.

**Deposit of Biological Materials**

The following biological materials have been deposited under the terms of the Budapest Treaty with the Agricultural Research Service Patent Culture Collection, Northern Regional Research Center (NRRL), Peoria, Illinois, and given the following accession numbers:

| Deposit | Accession Number | Date of Deposit |
|---|---|---|
| *E. coli* DH10B (pZL-ESA) | NRRL B-30068 | October 27, 1998 |
| *E. coli* DH10B (pZL-ESB) | NRRL B-30069 | October 27, 1998 |
| *E. coli* DH10B (pZL-ESC) | NRRL B-30070 | October 27, 1998 |

The strains have been deposited under conditions that assure that access to the cultures will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 C.F.R. §1.14 and 35 U.S.C. §122. The deposits represent substantially pure cultures of the deposited strains. The deposits are available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the

foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of conflict, the present disclosure including definitions will control.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.